Alteration of human hepatic drug transporter activity and expression by cigarette smoke condensate

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DOI: https://doi.org/10.1016/j.tox.2016.07.011

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PII: S0300-483X(16)30128-7
DOI: http://dx.doi.org/doi:10.1016/j.tox.2016.07.011
Reference: TOX 51708

To appear in: *Toxicology*

Received date: 9-6-2016
Revised date: 4-7-2016
Accepted date: 19-7-2016

Please cite this article as: Sayyed, Katia, Vee, Marc L.E., Abdel-Razzak, Ziad, Jouan, Elodie, Stieger, Bruno, Denizot, Claire, Parmentier, Yannick, Fardel, Olivier, Alteration of human hepatic drug transporter activity and expression by cigarette smoke condensate.Toxicology http://dx.doi.org/10.1016/j.tox.2016.07.011

Alteration of human hepatic drug transporter activity and expression by cigarette smoke condensate

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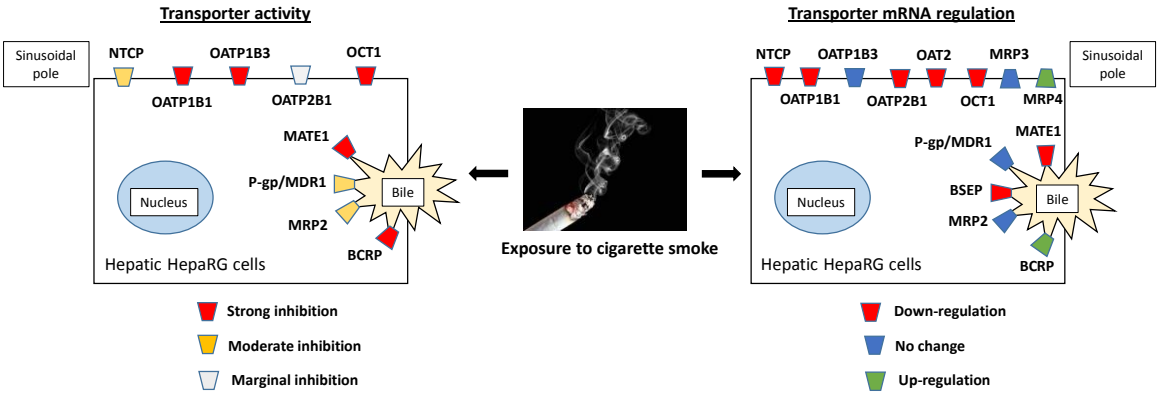
Graphical abstract



Summary

Smoking is well-known to impair pharmacokinetics, through inducing expression of drug metabolizing enzymes. In the present study, we demonstrated that cigarette smoke condensate (CSC) also alters activity and expression of hepatic drug transporters, which are now recognized as major actors of hepatobiliary elimination of drugs. CSC thus directly inhibited activities of sinusoidal transporters such as OATP1B1, OATP1B3, OCT1 and NTCP as well as those of canalicular transporters like P-glycoprotein, MRP2, BCRP and MATE1, in hepatic transporters-overexpressing cells. CSC similarly counteracted constitutive OATP, NTCP and OCT1 activities in human highly-differentiated hepatic HepaRG cells. In parallel, CSC induced expression of BCRP at both mRNA and protein level in HepaRG cells, whereas it concomitantly repressed mRNA expression of various transporters, including OATP1B1, OATP2B1, OAT2, NTCP, OCT1 and BSEP, and enhanced that of MRP4. Such changes in transporter gene expression were found to be highly correlated to those caused by 2,3,7,8-

tetrachlorodibenzo-p-dioxin, a reference activator of the aryl hydrocarbon receptor (AhR) pathway, and were counteracted, for some of them, by siRNA-mediated AhR silencing. This suggests that CSC alters hepatic drug transporter levels via activation of the AhR cascade. Importantly, drug transporter expression regulations as well as some transporter activity inhibitions occurred for a range of CSC concentrations similar to those required for inducing drug metabolizing enzymes and may therefore be hypothesized to be relevant for smokers. Taken together, these data established human hepatic transporters as targets of cigarette smoke, which could contribute to known alteration of pharmacokinetics and some liver adverse effects caused by smoking.

Abbreviations: ABC, ATP-binding cassette; AhR, aryl hydrocarbon receptor; ALDH3A1, aldehyde deshydrogenase 3A1; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; BSP, bromosulfophtalein; CCK8, cholecystokinin octapeptide sulphated; CF, carboxy-2.7-dichlorofluorescein; CSC, cigarette smoke condensate; CYP, cytochrome P-450; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; E3S, estrone-3-sulfate; EC_{50}, half maximal effective concentration; IC_{50}, half maximal inhibitory concentration; MATE, multidrug and toxin extrusion protein; MRP, multidrug resistance-associated protein; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NTCP, sodium-taurocholate co-transporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PAH, polycyclic aromatic hydrocarbon; P-gp, P-glycoprotein; PBS, phosphate-buffered saline; RT-qPCR; reverse transcription-quantitative polymerase chain reaction; SLC, solute carrier; TCDD, 2, 3, 7 ,8-tetrachlorodibenzo-p-dioxin; TEA, tetra-ethylammonium.

Key-words: Cigarette smoke; transporters; hepatocytes; drug-drug interactions; aryl hydrocarbon receptor.

1. Introduction

Cigarette smoking is well-known to cause many diseases, including lung cancer, heart disease and stroke (Ezzati et al. 2005; Sasco et al. 2004). By this way, tobacco smoke is recognized as one leading preventable cause of death (Jha 2009). In addition, smoking cause alterations of pharmacokinetics and drug interactions (Kroon 2007; Li and Shi 2015; Smith 2009). This has been primarily related to induction of liver detoxifying enzymes, including cytochrome P-450 (CYP) 1A1, CYP1B1, CYP1A2, CYP2B6 and glutathione S-transferases, by cigarette smoke chemicals (Chang et al. 2003; Eke and Iscan 2002; Schrenk et al. 1998; Washio et al. 2011). This results in increased metabolism of drugs like imipramine, meprobamate, oestrogens, pentazocine, phenylbutazone, theophylline and warfarin (Miller 1989). Higher doses of theophylline and some antipsychotics are consequently required to reach an optimal plasma concentration in smokers (Sohn et al. 2015).

Besides drug metabolizing enzymes, drug transporters, belonging to the solute carrier (SLC) or to the ATP-binding cassette (ABC) transporter families, constitute key-actors of the various steps of hepatic elimination of drugs (Giacomini et al. 2010; Kullak-Ublick et al. 2000). SLC transporters located at the sinusoidal pole of hepatocytes, such as organic anion transporting polypeptide (OATP/*SLCO*) 1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*), OATP2B1 (*SLCO2B1*), organic anion transporter (OAT) 2 (*SLC22A7*), sodium-taurocholate co-transporting polypeptide (NTCP/*SLC10A1*) and organic cation transporter (OCT) 1 (*SLC22A1*), are thus implicated in uptake of drugs from blood, *i.e.*, the so-called phase 0 of the hepatic drug detoxification system (van Montfoort et al. 2003). Transporters expressed at the canalicular

pole of hepatocytes, such as P-glycoprotein (P-gp), encoded by multidrug resistance gene 1 (*MDR1*/*ABCB1*), bile salt export pump (BSEP/*ABCB11*), multidrug resistance-associated protein (MRP/*ABCC*) 2 (MRP2/*ABCC2*), breast cancer resistance protein (BCRP/*ABCG2*) and multidrug and toxin extrusion protein (MATE) 1 (*SLC47A1*), are involved in secretion of drugs or drug metabolites into the bile, *i.e.*, the so-called phase 3a of liver detoxification (Funk 2008). Additionally, some sinusoidal ABC transporters like MRP3 (*ABCC3*) and MRP4 (*ABCC4*) can mediate back transport of drug metabolites into the blood for a secondary renal elimination, *i.e.*, the so-called phase 3b of the hepatic drug processing (Pfeifer et al. 2014).

Activity and/or expression of hepatic transporters have been shown to be regulated by a wide range of xenobiotics (Fardel et al. 2001), leading thus to drug-drug interactions, alterations of pharmacokinetics and liver toxicity (Li et al. 2014; Terada and Hira 2015). Interestingly, some chemicals highly present in cigarette smoke such as polycyclic aromatic hydrocarbons (PAHs) regulate expression of some hepatic transporters like BCRP (Ebert et al. 2005), MRP4 (Xu et al. 2010) and P-gp (Fardel et al. 1996; Mathieu et al. 2001). P-gp function is also targeted by tobacco smoke extracts in oral epidermal cells (Pan et al. 2009). Cigarette smoke extract additionally inhibits activity of the ABC transporter MRP1 (*ABCC1*) in lung epithelial cells (van der Deen et al. 2007). It is true that MRP1 is not, or only very faintly, present in hepatocytes (Payen et al. 2000), but this transporter shares many substrates and inhibitors with hepatic MRP2 (Keppler 2011). Taken together, these data suggest that cigarette smoke, that contains thousands of chemicals (Borgerding and Klus 2005), may interact with hepatic drug transporters in a notable way, as recently demonstrated for other air pollutants like diesel exhaust particles (Le Vee et al. 2015b). The present study was therefore designed to analyze regulation of hepatic drug transporter activity and expression in response to cigarette smoke condensate (CSC). Our data demonstrate that CSC can suppress activity of various SLC and

ABC transporters and also impairs expression of some of them. Such changes may participate to cigarette smoke-induced alteration of pharmacokinetics.

2. Materials and methods

2.1. Chemicals and reagents

CSC, supplied by Murty Pharmaceuticals (Lexington, KY), was prepared by smoking University of Kentucky's 3R4F standard research cigarettes on a Federal Trade Commission smoke machine (Nagaraj et al. 2006). Smoke particulates were collected on a glass fiber filter. The amount obtained was determined by weight increase of the filter and corresponded to a mean of 9.5 mg total particular matter/cigarette (Eldridge et al. 2015); nicotine represented 0.73 mg/cigarette, *i.e.*, 7.68 % (weight/weight) of total particular matter (Eldridge et al. 2015). CSC was finally prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO) to yield a 40 mg/mL solution. Verapamil, cyclosporine A, fumitremorgin C, probenecid, bromosulfophtalein (BSP), rifamycin SV, phenanthrene, nicotine, 4-aminobiphenyl, benzo(a)pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France), whereas carboxy-2.7-dichlorofluorescein (CF) diacetate and Hoechst 33342 were from Life Technologies (Villebon sur Yvette, France) and 2, 3, 7 ,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from Cambridge Isotope Laboratories (Cambridge, MA). [^{3}H(G)] taurocholic acid (sp. act. 5.0 Ci/mmol), [6,7-^{3}H(N)] estrone-3-sulfate (E3S) (sp. act. 54.0 Ci/mmol), [1-^{14}C] tetra-ethylammonium (TEA) (sp. act. 3.5 mCi/mmol) and [propionyl-^{3}H(N)] cholecystokinin octapeptide sulphated (CCK8) (sp. act. 101.5 Ci/mmol) were from Perkin-Elmer (Boston, MA). Mouse monoclonal antibodies against P-gp (clone C219), MRP2 (clone M2III-6), MRP4 (clone M4I-10) and BCRP (clone BXP21) were from Enzo Life Sciences (Villeurbanne, France), whereas mouse monoclonal antibody against heat shock cognate protein (HSC) 70 (clone B-6) was from Santa Cruz Biotechnology

(Dallas, TX). Mouse monoclonal antibody against aryl hydrocarbon receptor (AhR) (clone RPT1) was supplied by Abcam (Cambridge, United Kingdom), whereas rabbit polyclonal antibody raised against OATP2B1 has been previously described (Huber et al. 2007). All other chemicals and reagents were commercial products of the highest purity available.

2.2. Cell culture

Human highly-differentiated hepatoma HepaRG cells, which express most of hepatic drug transporters (Kotani et al. 2012; Le Vee et al. 2013) and are therefore well-recognized as surrogates of human hepatocytes in drug transporter studies (Bachour-El Azzi et al. 2015), were routinely cultured in Williams' E medium (Life Technologies) supplemented with 10% (vol/vol) fetal calf serum, 10 IU/mL penicillin, 10 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, and 5 x 10^{-5} M hydrocortisone hemisuccinate. Additional culture for two weeks in the same medium supplemented with 2% (vol/vol) DMSO was performed in order to get a full hepatocytic differentiation of the cells (Gripon et al. 2002).

P-gp overexpressing mammary MCF7R cells (Jouan et al. 2016) and MRP2-expressing human hepatoma HuH-7 cells (Olsavsky et al. 2007), were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies), supplemented with 10 % (vol/vol) fetal calf serum, 10 IU/mL penicillin and 10 µg/mL streptomycin. BCRP-transfected cells HEK 293 (HEK-BCRP cells) (Tournier et al. 2010), kindly donated by Dr X. Decleves (Faculty of Pharmacy, University Paris-Descartes, Paris, France), were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum, 100 IU/mL amoxicillin, 100 µg/mL erythromycin and 2 mg/mL G418.

HEK 293 cells overexpressing OATP1B1 (NM_006446) (HEK-OATP1B1 cells), OATP1B3 (NM_019844) (HEK-OATP1B3 cells), OATP2B1 (NM_007256) (HEK-OATP2B1 cells), NTCP (NM_003049) (HEK-NTCP cells), OCT1 (NM_003057) (HEK-OCT1 cells) or

MATE1 (NM_018242) (HEK-MATE1 cells) were prepared by transduction of HEK 293 cells by a lentiviral pLV-EF1-hOATP1B1-hPGK-GFP, pLV-EF1-hOATP1B3-hPGK-GFP, pLV-EF1-hOATP2B1-hPGK-GFP, pLV-EF1-hNTCP-hPGK-GFP, pLV-EF1-hOCT1-hPGK-GFP or pLV-EF1-hMATE1-hPGK-GFP, as previously described (Mayati et al. 2015). These transporter-transfected cells were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum, 10 IU/mL penicillin, 10 µg/mL streptomycin, 1% nonessential amino acids, and 1 µg/mL insulin.

2.3. Cytotoxicity assays

Cellular apoptosis or necrosis were investigated through cell staining with 10 µg/mL Hoechst 33342 and 1 µg/mL propidium iodide for 15 min at 37 °C, as previously described (Le Vee et al. 2014). Apoptotic cells, *i.e.*, cells with condensed blue chromatin or fragmented blue nuclei, and necrotic cells, *i.e.*, cells with red nuclei, were next counted in comparison with total cell population using fluorescence microscopy.

2.4. SLC transporter activity

SLC transporter activities were analyzed through determining intracellular accumulation of reference radiolabeled substrates of these SLC transporters at 37°c for 5 min (transporter-transfected HEK 293 cells) or 10 min (HepaRG cells), in the absence or presence of reference inhibitors or CSC, as previously described (Le Vee et al. 2015b; Mayati et al. 2015). The reference substrates were 40 nM taurocholate (for NTCP), 3.7 nM E3S (for OATP1B1 and OATP2B1), 2 nM CCK8 (for OATP1B3) and 28.6 µM TEA (for OCT1 and MATE1). The reference inhibitors were 100 µM BSP (for OATP1B1 and OATP2B1), 10 µM rifamycin SV (for OATP1B3) and verapamil (50 µM for OCT1 and 100 µM for MATE1); reference inhibition of NTCP was achieved through withdrawal of sodium (Le Vee et al.

2015a). Incubations with substrates were performed in a well-defined medium at pH adjusted to 7.4 (Le Vee et al. 2013), except for MATE1 for which the pH was set at 8.4 (Mayati et al. 2015). After washing with phosphate-buffered saline (PBS), cells were finally lysed and intracellular accumulation of substrates was determined by scintillation counting and normalized to total protein content, determined by the Bradford method (Bradford 1976). Data were expressed as % of substrate accumulation in control cells not exposed to inhibitor or CSC. Data were also alternatively expressed as % of transporter activity found in control cells, arbitrarily set at 100%, according to the following equations:

For OATP1B1, OATP1B3, OCT1 and MATE1:

$$\%\ \text{SLC transporter activity} = \frac{(\text{Accumulation}_{+CSC} - \text{Accumulation}_{+\text{reference inhibitor}}) \times 100}{\text{Accumulation}_{Control} - \text{Accumulation}_{+\text{reference inhibitor}}}$$

with $\text{Accumulation}_{+CSC}$ = substrate accumulation in the presence of CSC, $\text{Accumulation}_{control}$ = substrate accumulation in control cells and $\text{Accumulation}_{+\text{refrence inhibitor}}$ = substrate accumulation in the presence of a reference transporter inhibitor.

For NTCP:

$$\%\ \text{NTCP activity} = \frac{(\text{Accumulation}_{+CSC} - \text{Accumulation}_{Control/-Na^+}) \times 100}{\text{Accumulation}_{Control/+Na^+} - \text{Accumulation}_{Control/-Na^+}}$$

with $\text{Accumulation}_{+CSC}$ = taurocholate accumulation in the presence of CSC and sodium, $\text{Accumulation}_{control/+Na^+}$ = taurocholate accumulation in control cells in the presence of sodium and $\text{Accumulation}_{control/-Na^+}$ = taurocholate accumulation in control cells in the absence of sodium.

2.5. ABC transporter activity

ABC transporter activities were analyzed through measuring intracellular accumulation or retention of fluorescent substrates of P-gp, MRP2 or BCRP, in the absence or presence of reference inhibitors or CSC, as previously described (Fardel et al. 2015; Le Vee et al. 2015b).

Briefly, for P-gp and MRP2 activities, P-gp-expressing MCF7R cells and MRP2-expressing HuH-7 cells were incubated with 5.25 µM rhodamine 123 (P-gp substrate) or 3 µM diacetate ester of CF (MRP2 substrate) for 30 min at 37°C , in the presence or absence of 100 µM cyclosporine A (P-gp inhibitor), 2 mM probenecid (MRP2 inhibitor) or CSC. After washing with PBS, cells were lysed and intracellular accumulation of fluorescent dyes was determined by spectrofluorimetry (excitation and emission wavelengths were 485 and 535 nm, respectively) using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA). Data were normalized to total protein content and were expressed as % of dye accumulation in control cells not exposed to reference inhibitor or CSC. Data were also alternatively expressed as % of transporter activity found in control cells, arbitrarily set at 100%, according to the following equation:

$$\%\ \text{P-gp or MRP2 activity} = \frac{(\text{Accumulation}_{+\text{reference inhibitor}} - \text{Accumulation}_{+\text{CSC}}) \times 100}{\text{Accumulation}_{+\text{reference inhibitor}} - \text{Accumulation}_{\text{Control}}}$$

with $\text{Accumulation}_{+\text{CSC}}$ = substrate accumulation in the presence of CSC, $\text{Accumulation}_{\text{control}}$ = substrate accumulation in control cells and $\text{Accumulation}_{+\text{reference inhibitor}}$ = substrate accumulation in the presence of a reference transporter inhibitor.

For BCRP activity, HEK-BCRP cells were first loaded at 37°C with 16.2 µM Hoechst 33342 (BCRP substrate) for 30 min. After washing in PBS, cells were re-incubated in Hoechst 33342-free medium at 37°C for 90 min in the absence or presence of 10 µM fumitremorgin C (BCRP inhibitor) or CSC. After washing in PBS, cells were lysed and intracellular retention of Hoechst 33342 was next determined by spectrofluorimetry (excitation and emission wavelengths were 355 and 460 nm, respectively). Data were normalized to total protein content and were expressed as % of dye retention in control cells not exposed to fumitremorgin C or CSC. Data were also alternatively expressed as % of transporter activity found in control cells, arbitrarily set at 100%, according to the following equation:

$$\% \text{ BCRP activity} = \frac{(\text{Retention}_{+\text{Fumitremorgin C}} - \text{Retention}_{+\text{CSC}}) \times 100}{\text{Retention}_{+\text{Fumitremorgin C}} - \text{Retention}_{\text{Control}}}$$

with $\text{Retention}_{+\text{CSC}}$ = Hoechst 33342 retention in the presence of CSC, $\text{Retention}_{\text{control}}$ = Hoechst 33342 retention in control cells and $\text{Retention}_{+\text{Fumitrtemorgin C}}$ = Hoechst 33342 retention in the presence of fumitremorgin C.

2.6. RNA isolation and analysis

Total RNAs were extracted using the TRI reagent (Sigma-Aldrich). RNA was then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using the RT kit from Applied Biosystems (Foster City, CA), the fluorescent dye SYBR Green methodology and a CFX384 real-time PCR system (Bio-Rad, Hercules, CA), as previously described (Le Vee et al. 2013). Gene-specific primers for drug transporters, CYP1A1, CYP1B1, aldehyde deshydrogenase 3A1 (ALDH3A1) and 18 S rRNA were exactly as previously reported (Le Vee et al. 2010; Le Vee et al. 2013). The specificity of each gene amplification was verified at the end of qPCR reactions through analysis of dissociation curves of the PCR products. Amplification curves were next analysed with CFX Manager software (Bio-Rad), using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to the 18S rRNA endogenous reference using the $2^{(-\Delta Ct)}$ method. Data were finally expressed as fold of values found in untreated control cells, arbitrarily set at the value of 1 unit.

2.7. RNA interference experiments

Control non-targeting siRNAs (siNT) or siRNAs targeting AhR (siAhR), provided by Sigma-Aldrich and initially prepared in Opti-MEM medium (ThermoFischer Scientific, Waltham, MA) at a final 0.4 µM concentration, were diluted into the transfection reagent DharmaFECT 1 (Dharmacon, Lafayette, CO) and next incubated overnight at 37°c with

HepaRG cells plated in 24 wells plates (approximately 450000 cells/well). Transfected HepaRG cells were then maintained with Williams' E medium, supplemented with 2% (vol/vol) DMSO, 10% (vol/vol) fetal calf serum, 5 µg/ml insulin, 10 IU/ml penicillin, 10 µg/ml streptomycin, 2 mM glutamine, and 5×10^{-5} M hydrocortisone hemisuccinate for 24 h before CSC treatment.

2.8. Western blot analysis

Total protein extracts were prepared from HepaRG cells as previously reported (van Grevenynghe et al. 2004). Proteins were then separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking in Tris-buffered saline containing 4% bovine serum albumin, membranes were incubated overnight at 4°C with primary antibodies directed against transporters or AhR or against HSC70, used here as a loading control. Peroxidase-conjugated antibodies were thereafter used as secondary antibodies. After washing, immunolabeled proteins were visualized by chemiluminescence. Intensities of antibody-stained bands were finally measured by densitometry using ImageJ 1.40g software (National Institute of Health, Besthesda, MA), allowing to normalize anti-transporter or anti-AhR antibodies-related staining to anti-HCS70 antibody-labeling.

2.8. Statistical analysis

Quantitative data were usually expressed as means ± SEM. Data were statistically analyzed using Student's *t* test, analysis of variance (ANOVA) followed by Dunnett's or Newman-Keuls post-hoc test or the nonparametric Spearman's rank correlation method. The criterion of significance was $p < 0.05$. Half maximal inhibitory concentration (IC_{50}) and half maximal effective concentration (EC_{50}) values were determined using GraphPad Prism

software (GraphPad Software, La Jolla, CA), through nonlinear regression based on the four parameter logistic function.

3. Results

3.1. Effects of CSC on SLC transporters activities.

The effects of 320 µg/mL CSC, a concentration close to those previously retained in *in vitro* studies (Allam et al. 2013; Cohen et al. 2009), on SLC transporter activities was first analyzed in various HEK 293 clones overexpressing hepatic SLC transporters. As shown in Fig. 1A, 320 µg/mL CSC markedly inhibited cellular uptake of reference substrates for OATP1B1 (E3S), OATP1B3 (CCK8), NTCP (taurocholate), OCT1 (TEA) and MATE1 (TEA). These inhibitions of SLC transporter activities triggered by CSC were similar to those caused by reference inhibitors (BSP for OATP1B1, rifamycin SV for OATP1B3 and verapamil for OCT1 and MATE1) or to withdrawal of sodium (for NTCP, whose activity is driven by sodium gradient) (Fig. 1A). CSC used at 320 µg/mL also inhibited OATP2B1-mediated uptake of E3S, but only in a minor way when compared to the reference inhibitor BSP (Fig. 1A), thus ruling out the hypothesis that the marked inhibitory effect of CSC towards other SLC transporters described above may be due to a non-specific toxicity of 320 µg/mL CSC towards HEK 293 cells.

To assess CSC IC_{50} towards SLC transporters that were markedly inhibited, *i.e.*, OATP1B1, OATP1B3, NTCP, OCT1 or MATE1, dose-responses of CSC effects towards their activities were next characterized. As indicated in Fig. 1B, OATP1B1, OATP1B3, OCT1 and MATE1 were sensitive to relatively low concentrations of CSC, around 10 µg/mL, with IC_{50} values ranging from 6.1 µg/mL (for MATE1) to 12.5 µg/mL (for OCT1). By contrast, much higher concentrations of CSC were needed to inhibit NTCP activity, with an IC_{50} value of 140.0 µg/mL (Fig. 1B).

Effects of CSC on constitutive NTCP, OATP and OCT1 activities exhibited by HepaRG cells, that are well-differentiated hepatoma cells displaying functional expression of main uptake SLC transporters in a hepatic configuration (Le Vee et al. 2013), were further analyzed. CSC, used at 320 µg/mL, was found to markedly inhibit cellular uptake of the OATP substrate E3S, of the NTCP substrate taurocholate, and of the OCT1 substrate TEA (Fig. 2). These inhibitory effects of CSC in HepaRG cells were found to be similar to those caused by blocking the transporters through the use of reference inhibitors (BSP for OATPs and verapamil for OCT1) or the withdrawal of sodium (for NTCP) (Fig. 2).

The effects of some major reference components of CSC, *i.e.*, nicotine, NNK, benzo(a)pyrene, phenanthrene and 4-aminobiphenyl (van Leeuwen et al. 2005), on activities of OATP1B1 and OCT1, selected here as prototypical organic anion or cation transporters targeted by CSC, were finally characterized. As shown in Fig. 3, the PAHs benzo(a)pyrene and phenanthrene, used either at 10 or 100 µM, failed to impair uptake of E3S and TEA in HEK-OATP1B1 and HEK-OCT1 cells, respectively. Similarly, NNK did not inhibit accumulation of TEA; this compound however enhanced uptake of E3S in HEK-OATP1B1 cells when used at 100 µM, thus suggesting that this NNK concentration may *cis*-stimulate OATP1B1 activity. Nicotine used at 100 µM was found to decrease uptake of TEA in HEK-OCT1 cells (Fig. 3); by contrast, this compound, used either at 10 or 100 µM, failed to alter E3S accumulation in HEK-OATP1B1 cells. The chemical 4-aminobiphenyl, used at 100 µM, decreased accumulation of E3S and OCT1 in HEK-OATP1B1 and HEK-OCT1 cells, respectively; when used at a lower concentration (10 µM), 4-aminobiphenyl however did not impair accumulation of E3S and TEA (Fig. 3).

3.2. Effects of CSC on ABC transporters activities.

CSC, used at 320 µg/mL, enhanced accumulation of the P-gp substrate rhodamine 123 in P-gp-overexpressing MCF7R cells and of the MRP substrate CF in MRP2-expressing HuH-7 cells (Fig. 4A), thus indicating that CSC most likely inhibits the efflux of dyes mediated by P-gp or MRP2. Accumulation levels of the fluorescent substrates were also increased by reference inhibitors of ABC transporters, *i.e.*, cyclosporine A for P-gp and probenecid for MRP2 (Fig. 4A). 320 µg/mL CSC, like the BCRP reference inhibitor fumitremorgin C, additionally enhanced intracellular retention of the BCRP substrate Hoechst 33342 in HEK-BCRP cells (Fig. 4A), thus supporting the conclusion that CSC blocks BCRP efflux activity. Inhibitory effects of CSC towards P-gp, MRP2 and BCRP were shown to be dose-dependent, with IC_{50} values ranging from 37.4 µg/mL (for BCRP), to 197.4 µg/mL (for MRP2) and 224.3 µg/mL (for P-gp) (Fig. 4B).

3.3. Regulation of hepatic drug transporters genes expression by CSC.

To analyze the potential effects of CSC on hepatic drug transporter expression, human hepatic HepaRG cells were exposed to 40 µg/mL CSC for 48 h. This CSC concentration was not cytotoxic to HepaRG cells as demonstrated by the analysis of apoptotic/necrotic cells using Hoechst 33342/propidium iodide assay (data not shown) and is in the range of CSC concentrations known to affect gene expression in various types of cultured cells, including human hepatic cells (Fields et al. 2005; Nagaraj et al. 2006; Xiao et al. 2015). Exposure of HepaRG cells to this CSC concentration markedly enhanced mRNA expression of CYP1A1, CYP1B1 and ALDH3A1 (Fig. 5A), that are well-known to respond to cigarette smoke (Spira et al. 2004), thus indicating that CSC was fully active in our experimental exposure conditions.

CSC was found to significantly repress mRNA expression of various hepatic transporters like the SLC transporters OATP1B1, OATP2B1, OAT2, NTCP, OCT1 and MATE1 and the ABC transporter BSEP (Fig. 5B). By contrast, it increased mRNA levels of

MRP4 and BCRP, whereas mRNA expression of other transporters such as OATP1B3, MDR1, MRP2 and MRP3 remained unchanged (Fig. 5B). The effects of CSC towards mRNA repression of some transporters were demonstrated to be dose-dependent, with IC_{50} values around 15-20 µg/mL for most of repressed transporters such as OAT2, NTCP, OCT1 and BSEP and around 40 µg/mL for OATP2B1 (Fig. 6). Induction of BCRP mRNA by CSC was also dose-dependent, with an EC_{50} value of 25.2 µg/mL, in the range of those found for CYP1A1 (EC_{50}=14.3 µg/mL) and CYP1B1 (EC_{50}=43.0 µg/mL) mRNA induction in CSC-treated HepaRG cells (Fig. 6). Besides mRNA level, protein expression of some transporters was also impaired by CSC. CSC thus markedly repressed OATP2B1 protein level and induced that of BCRP in HepaRG cells (Fig. 7). CSC however failed to significantly alter expression of P-gp, MRP2 and MRP4 at the protein level (Fig. 7).

3.4. Contribution of AhR to CSC-mediated regulation of hepatic drug transporters expression

AhR is a ligand-activated transcription factor, well-known to be implicated in gene regulation by cigarette smoke (Gebremichael et al. 1996; Kitamura and Kasai 2007). To analyze its potential role in CSC-mediated regulation of hepatic drug transporters, we first compared the effects of CSC toward transporter mRNA levels to those caused by TCDD, a reference agonist for AhR (Sorg 2014). As indicated in Fig. 8A, exposure to 10 nM TCDD for 48 h decreased mRNA expressions of OATP1B1, OATP2B1, OAT2, NTCP, OCT1, BSEP and MATE1, whereas it significantly enhanced those of MRP3, MRP4 and BCRP. Transporters were next ranked from the most induced to the most repressed according to their mRNA expression level in TCDD- or CSC-treated HepaRG cells; potential correlation between the transporter regulation profiles was then analyzed using the Spearman's rank correlation. As shown in Fig. 8B, the global effect of CSC exposure on drug transporter expression in HepaRG

cells was found to be highly correlated to that of TCDD, suggesting that CSC and TCDD may share the same signaling way for regulating transporter levels, *i.e.*, the AhR signaling pathway.

The consequences of AhR expression extinction on drug transporter regulation by CSC were next determined. In agreement with previous data (Le Vee et al. 2010), HepaRG cells transfected with siRNAs against AhR exhibited a marked down-regulation of AhR protein expression in HepaRG cells (Fig. 9A); they concomitantly displayed a huge reduction of CYP1A1 and CYP1B1 mRNA induction in response to CSC (Fig. 9B), thus demonstrating that siRNA-mediated AhR knock-down resulted in efficient suppression of the AhR signaling pathway in hepatic HepaRG cells. With respect to transporters, AhR silencing significantly counteracted the repressing effects of CSC towards OAT2 and OCT1 mRNA levels (Fig 9B). Repression of OATP2B1 mRNA expression by CSC was also attenuated by AhR knock-down, knowing that the level of statistical significance was however not reached (Fig. 9B). Transfection of siRNAs against AhR concomitantly and significantly abrogated CSC-mediated induction of MRP4 and BCRP mRNA expression, whereas CSC-triggered down-regulation of NTCP and BSEP were not impacted (Fig. 9B).

4. Discussion

Previous studies have demonstrated that cigarette smoke can regulate detoxifying enzymes in hepatic and extra-hepatic tissues, thus causing alteration of pharmacokinetics and drug interactions (Kroon 2007; Li and Shi 2015). The data reported in the present work demonstrate that CSC can alter activity and/or expression of hepatic drug transporters (See Table 1 for a summary of the effects of CSC towards transporter activity and expression), thus fully highlighting that drug transporters also constitute molecular targets for cigarette smoke chemicals.

Activities of sinusoidal uptake transporters such as OATP1B1, OATP1B3, NTCP and OCT1 as well as those of the canalicular transporters P-gp, MRP2, BCRP and MATE1 were thus inhibited by CSC. IC_{50} values however differ according to transporters, allowing to discriminate two groups of transporters: one, comprising OATP1B1, OATP1B3, OCT1, MATE1 and BCRP, corresponds to transporters strongly inhibited by CSC, with IC_{50} values in 6-37 µg/mL range, and the other, comprising NTCP, P-gp and MRP2, to hepatic transporters more moderately inhibited by CSC, with IC_{50} values in 140-224 µg/mL range (Table 1). This difference in sensitivity of transporters to CSC, associated to the fact that at least activity of one hepatic transporter, *i.e.*, OATP2B1, was only marginally impacted by CSC, discards the hypothesis of a general and non-discriminating inhibitory effect of CSC towards membrane transporter activities. Inhibitory effects of CSC towards hepatic drug transporter can rather be considered as specific, most likely reflecting direct and transporter-dependent interactions of CSC chemicals with substrate and/or regulatory binding sites on drug transporters, as classically thought for drug transporter inhibitors (Montanari and Ecker 2015). The exact nature of CSC chemicals responsible for transporter inhibition remains to be determined, knowing that it may differ according to transporters. Hydrophilic and/or hydrophobic nonvolatile components of cigarette smoke particles are likely to be involved, because CSC, that represents total particulate matter of smoke (Johnson et al. 2009), contains such chemicals, whereas cigarette smoke extract generated by bubbling cigarette smoke through culture media corresponds to only water-soluble smoke chemicals (Muller and Gebel 1998). With respect to inhibition of OATP1B1 and OCT1, a major role for PAHs, that constitute major toxic components of cigarette smoke (Rodgman et al. 2000), can likely be excluded owing to the fact that both benzo(a)pyrene and phenanthrene failed to alter E3S and TEA uptake in HEK-OATP1B1 and HEK-OCT1 cells, respectively. An implication of CSC-contained PAHs in MRP2 activity inhibition can similarly be discarded because various PAHs, including benzo(a)pyrene, phenanthrene, benzo[b]fluoranthene and

chrysene as well as the nitro-PAH 1-nitropyrene, failed to alter MRP2 activity (Le Vee et al. 2015b). A role for NNK in inhibition of OATP1B1 and OCT1 seems also at first view unlikely because this chemical failed to decrease E3S and TEA uptake mediated by these SLC transporters. The fact that NNK *cis*-stimulated E3S uptake in HEK-OATP1B1 cells however indicates that this compound can interact with OATP1B1 activity. *Cis*-stimulation of OATP transporters has already been reported for other chemicals (De Bruyn et al. 2013); the molecular features that determine such a *cis*-stimulatory effect remain however very poorly characterized. Nicotine, which contributes in a major way to the addictive properties of tobacco smoking (Zaniewska et al. 2009), was found to inhibit OCT1 activity, in agreement with previous data (Urakami et al. 1998); by contrast, this organic cation failed to alter OATP1B1 activity. A participation of nicotine to inhibitory effect of CSC towards OCT1 activity may therefore be considered. In this context, it is noteworthy that nicotine effects on OCT1 activity were dose-dependent and the 10 µM nicotine concentration, which is much higher than blood nicotine concentrations in individual smokers, that varied from 25 to 444 nM (Russell et al. 1980), was inactive on OCT1 activity. *In vivo* concentrations of nicotine in smokers are therefore very unlikely to contribute alone to inhibition of OCT1 activity. The same conclusion may be drawn for the carcinogenic aromatic amine 4-aminobiphenyl, that blocked OCT1 activity, and also that of OATP1B1, but only when used at a high 100 µM concentration. It should however be kept in mind that CSC contains probably thousands of chemicals (Borgerding and Klus 2005) and additive or synergic effects of some of these chemicals, including nicotine and 4-aminobiphenyl, may be involved in inhibition of transporter activity by CSC. Moreover, the role of putative metabolites formed from CSC chemicals may have also to be considered.

Besides directly inhibiting drug transporter activity, CSC also modulated expression of hepatic drug transporters. Some of them were up-regulated, especially the canalicular ABC efflux BCRP, induced by CSC at both mRNA and protein level in HepaRG cells (Table 1). The

sinusoidal ABC efflux pump MRP4 was also up-regulated at the mRNA level, but not at the protein level (Table 1); such a discrepancy may be due to the relative weak mRNA induction of this transporter by CSC or, alternatively, to a divergent regulation between transcriptional and post-transcriptional level, as already described for cytokine- or diesel exhaust particle extract-mediated regulation of hepatic transporter expression (Le Vee et al. 2015b; Le Vee et al. 2009). CSC repressed mRNA expression of other transporters, such as the SLC transporters OATP1B1, OATP2B1, OAT2, NTCP and MATE1 and the canalicular ABC transporter BSEP; OATP2B1 protein expression was in parallel markedly decreased by CSC. By contrast, expression of the canalicular ABC efflux pumps P-gp and MRP2 were not altered by CSC treatment, both at the mRNA and protein levels; mRNA level of the sinusoidal ABC pump MRP3 was similarly not impacted. Overall, these CSC-induced changes in hepatic transporter expression correspond to a repression of most of sinusoidal SLC uptake transporters and a preservation or an induction of main ABC efflux pumps. They may consequently be interpreted as a protective mechanism of hepatic cells exposed to CSC-containing chemicals, possibly leading to decreased intracellular accumulation of potential toxic CSC chemicals through reduction of their uptake and enhancement of their efflux. BCRP induction by CSC likely argues in favor of this hypothesis because BCRP up-regulation may result in enhanced efflux of toxic PAH metabolites handled by this efflux pump (Ebert et al. 2005).

AhR, a well-recognized xenobiotic-sensing receptor activated by CSC-contained chemicals such as PAHs (Gebremichael et al. 1996; Kitamura and Kasai 2007), most likely plays a major role in CSC-mediated regulation of transporter expression. Indeed, the AhR signaling pathway was activated in CSC-exposed HepaRG cells, as demonstrated by up-regulation of the reference AhR target genes CYP1A1, CYP1B1 and ALDH3A1. This AhR-related induction of CYP1A1 and CYP1B1 genes occurred for CSC concentrations in the 14-43 µg/mL range, similar to the range of CSC concentrations (14-39 µg/mL) acting on

transporter expression. This suggests a common signaling pathway, *i.e.*, the AhR cascade, for CYP1A1/1B1 and transporter regulation by CSC. This hypothesis is reinforced by the fact that the profile of transporters expression changes in CSC-exposed HepaRG cells is highly correlated to that resulting from treatment by the reference AhR agonist TCDD. Moreover, siRNA-mediated knock-down of AhR expression fully supported a major implication of AhR in CSC-mediated mRNA regulation of several hepatic transporters, notably OAT2, OCT1, BCRP and MRP4, knowing that BCRP and MRP4 are already known to be targeted by the AhR cascade (Ebert et al. 2005; Tompkins et al. 2010; Xu et al. 2010). AhR-unrelated signaling ways may however additionally participate to transporter regulation by CSC, notably for CSC-mediated-repressions of the bile acid transporters NTCP and BSEP, which were not counteracted by AhR knock-down.

The relevance of our *in vitro* findings to *in vivo* exposure to cigarette smoke chemicals constitutes probably a key-point that has to be clarified. It is unfortunately rather difficult to relate an *in vitro* exposure of cultured cells to CSC to a dose delivered *in vivo* to smokers. On the one hand, CSC concentrations (around 10-40 µg/mL) regulating drug transporter expression (Fig. 6) and inhibiting some transporters like OATP1B1, OATP1B3, OCT1 and MATE1 (Fig. 1B) are similar to those used in previous studies with cultured cells, including human hepatic cells (Fields et al. 2005; Nagaraj et al. 2006; Xiao et al. 2015); they also up-regulated the drug metabolizing enzymes CYP1A1 and CYP1B1, that, together with other AhR targets such as CYP1A2, constitute key targets induced by smoking in humans (Chang et al. 2003; Dobrinas et al. 2011; Thum et al. 2006) and are in the range of CSC concentrations (20 to 120 µg/mL) previously hypothesized to be relevant for human smokers (Gao et al. 2005). On the other hand, 10-40 µg/mL CSC corresponds to 0.768-3.072 µg/mL (4.73-18.92 µM) nicotine, when considering that CSC contains 7.68 % (weight/weight) nicotine (Eldridge et al. 2015), and such nicotine concentrations are much higher than those (25 to 444 nM) commonly described in

smoker blood (Russell et al. 1980). This may indirectly favor the hypothesis that 10-40 µg/mL CSC acting on transporters may not be reached in smokers, even if toxicokinetics features of nicotine and those of yet unidentified cigarette smoke chemicals targeting transporters may differ in a major way, bringing caution towards any extrapolation based on nicotine disposition. Otherwise, it should be kept in mind that cell culture models often do not exhibit all the differentiated and functional characteristics of the corresponding native epithelium or the entire organ, notably with respect to expression of antioxidant enzymes and glutathione level; *in vitro* treatment with CSC may therefore not exactly replicate *in vivo* responses to smoke (Xiao et al. 2015). Additional studies should be consequently required to precisely determine whether our *in vitro* CSC concentrations are relevant for *in vivo* liver exposure to cigarette smoke chemicals.

Another key point that remains to be determined corresponds to the potential deleterious consequences of smoking-related modulation of hepatic transporter activity or expression. Inhibition of OATP1B1 and OATP1B3 activity associated to repression of OATP2B1 expression by cigarette smoke chemicals may impair hepatic uptake of drug substrates for OATP transporters such as statins. Alteration of statin pharmacokinetics by smoking has however yet not been reported, according to the best of our knowledge, even if smoking diminishes the beneficial effect of statins (Milionis et al. 2001). Hepatobiliary elimination of hormones substrates for OATPs, such as steroid and thyroid hormones, may also be hypothesized to be impaired by putative OATP inhibition in smokers, which may contribute to endocrine disruption caused by smoking (Windham et al. 2005). Finally, repression of the bile acid transporter BSEP by cigarette smoke components may be suspected to alter bile secretion and such a cholestatic effect may participate to the well-established liver toxicity of smoking (Corpechot et al. 2012; Zein 2010).

In summary, CSC was shown to act as a bifunctional modulator of hepatic drug transporters, *i.e.*, it inhibits their activity and alters expression of some of them. Such effects of

cigarette smoke chemicals may contribute to alterations of pharmacokinetics or some adverse effects caused by smoking.

Acknowledgments

KS was supported by a grant from AZM Association-UL (Tripoli, Lebanon).

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Legends to figures

Fig. 1. Effects of CSC on hepatic SLC transporter activities.

(A) SLC transporter-overexpressing HEK 293 cells were incubated for 5 min with reference radiolabeled substrates (E3S for OATP1B1 and OATP2B1, CCK8 for OATP1B3, taurocholate (TC) for NTCP and TEA for OCT1 and MATE1) in the absence (control/CTR) or the presence of 320 µg/mL CSC; reference inhibitions of transporter activities were obtained in parallel through addition of 100 µM BSP (for OATP1B1 and OATP2B1), 10 µM rifamycin SV (RIFSV) (for OATP1B3), 50 µM verapamil (VRP) (for OCT1) or 100 µM verapamil (MATE1) or withdrawal of sodium (NTCP). Intracellular accumulations of substrates were then determined by scintillation counting. Data are expressed as percentage of substrate uptake in control cells and are the means $\pm$ SEM of at least three independent assays. *, $p<0.05$ when compared to control cells. (B) SLC transporter-overexpressing cells were incubated with radiolabeled substrates in the absence or presence of various CSC concentrations; reference inhibitions of transporter activities were done in parallel as described above. SLC transporter activities were then calculated as described in Materials and Methods and are expressed as percentage of those found in control cells not exposed to CSC, arbitrarily set at 100%. Data are the means $\pm$ SEM of at least three independent assays. CSC IC_{50} values are indicated on the top of each graph.

Fig. 2. Effect of CSC on OATP, NTCP and OCT1 activities in human hepatoma HepaRG cells.

HepaRG cells were incubated for 10 min with reference radiolabeled substrates (E3S for OATPs, taurocholate (TC) for NTCP and TEA for OCT1) in the absence (control/CTR) or the presence of 320 µg/mL CSC; reference inhibitions of transporter activities were obtained in parallel through addition of 100 µM BSP (OATPs) or 50 µM verapamil (VRP) (OCT1) or

withdrawal of sodium (NTCP). Intracellular accumulations of substrates were then determined by scintillation counting. Data are expressed as percentage of substrate uptake in control cells and are the means ± SEM of at least three independent assays. *, $p<0.05$ when compared to control cells.

Fig. 3. Effects of some cigarette smoke chemical components on OATP1B1 and OCT1 activities.

HEK-OATP1B1 and HEK-OCT1 cells were incubated for 5 min with reference radiolabeled substrates (E3S for OATP1B1 and TEA for OCT1) in the absence (control/CTR) or the presence of chemical components of cigarette smoke (nicotine, NNK, benzo(a)pyrene, phenanthrene and 4-aminobiphenyl), used at 10 and 100 µM, or of reference inhibitors (100 µM BSP for OATP1B1 and 50 µM verapamil (VRP) for OCT1). Intracellular accumulations of substrates were next determined by scintillation counting. Data are expressed as percentage of substrate uptake in control cells and are the means ± SEM of at least three independent assays. *, $p<0.05$ when compared to control cells.

Fig. 4. Effects of CSC on hepatic ABC transporter activities.

(A) P-gp, MRP2 and BCRP activities were determined through measurement of rhodamine 123 accumulation, CF accumulation and Hoechst 33342 retention in ABC transporter-expressing cell lines, *i.e.*, MCF7R (for P-gp activity), HuH-7 (for MRP2 activity) and HEK-BCRP (for BCRP activity), in the absence (control/CTR) or presence of 320 µg/mL CSC, as reported in the Material and Methods. Reference inhibitions of transporter activities were obtained in parallel through addition of 100 µM cyclosporine A (CSA) (P-gp inhibition), 2 mM probenecid (PBN) (MRP2 inhibition) or 10 µM fumitremorgin C (FTC) (BCRP inhibition). Data are expressed as percentage of dye accumulation (P-gp and MRP2 activities) or dye retention

(BCRP activity) found in control untreated cells; they are the means ± SEM of at least three independent experiments. *, $p<0.05$ when compared to control cells. (B) P-gp, MRP2 and BCRP activities were determined in ABC transporter-expressing cells as described above, in the absence or presence of various concentrations of CSC or of reference inhibitors. ABC transporter activities were next calculated as described in Materials and Methods and are expressed as percentage of those found in control cells not exposed to CSC, arbitrarily set at 100%. Data are the means ± SEM of at least three independent assays. CSC IC_{50} values are indicated on the top of each graph.

Fig. 5. Regulation of hepatic drug transporter mRNA expression by CSC.

Human highly-differentiated hepatoma HepaRG cells were either untreated or exposed to 40 µg/mL CSC for 48 h. (A) CYP1A1, CYP1B1 and ALDH3A1 and (B) drug transporter mRNA expression was next analyzed by RTqPCR. Data are expressed as fold factor of mRNA expression found in untreated cells, indicated by a dotted line, and are the means ± SEM of four independent assays. *, $p<0.05$ when compared to control untreated cells.

Fig. 6. Concentration-dependent effects of CSC on drug transporter mRNA expression.

Differentiated HepaRG cells were either untreated or exposed to various concentrations of CSC (from 0.1 to 160 µg/mL) for 48 h. (A) CYP1A1, CYP1B1 and drug transporter mRNA expression was next analyzed by RTqPCR. Data are expressed as fold factor of mRNA expression found in untreated cells and are the means ± SEM of four independent assays. *, $p<0.05$ when compared to control untreated cells.

Fig. 7. Regulation of hepatic drug transporter protein expression by CSC.

HepaRG cells were either untreated or exposed to 40 µg/mL CSC for 48 h. Transporter protein levels were then determined by Western-blot analysis. (A) A representative blot is shown for each transporter. (B) For each transporter, data were quantified by densitometric analysis, normalized to HSC70 staining and expressed relative to transporter expression found in untreated cells, arbitrarily set at the value of 100% and indicated by a dotted line; they are the means ± SEM of values from three independent assays. *, $p<0.05$ when compared to untreated cells.

Fig. 8. Correlation of CSC- and TCDD-induced changes of drug transporter mRNA levels.

(A) HepaRG cells were either untreated or exposed to 10 nM TCDD for 48 h. Drug transporter mRNA expression was next evaluated by RT-qPCR. Data are expressed as percentage of transporter expression found in control untreated cells, arbitrarily set at the value of 100% and indicated by a dotted line; they are the means ± SEM of three independent assays. *, $p<0.05$ when compared to untreated control cells. (B) Drug transporters were ranked according to regulation of their mRNA expression in response to a 48-h treatment by 40 µg/mL CSC or 10 nM TCDD. For this purpose, transporters were ranked for each treatment from the most induced transporter to the most repressed, from data from Fig. 5B and Fig. 8A. Correlation was analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and p values are provided on the top of the correlation graph.

Fig. 9. Effects of AhR silencing on CSC-mediated regulation of drug transporter mRNA expression.

HepaRG cells were transfected with siRNAs against AhR (siAhR) or with non-targeting siRNA (siNT). (A) AhR expression was then determined by Western-blot analysis. Left panel, a representative blot is shown. Right panel, AhR expression was quantified by densitometric

analysis, normalized to HCS70 staining and expressed relative to AhR expression found in siNT-transfected cells, arbitrarily set at the value of 100%; the results are the means ± SEM of values from three independent assays. *, $p<0.05$ when compared to untreated cells. (B) siAhR- and siNT-transfected HepaRG cells were either untreated (control/CTR) or treated by 40 µg/mL CSC for 48 h. CYP1A1, CYP1B1 and drug transporter mRNA expression was then determined by RT-qPCR. Data are expressed as fold factor comparatively to mRNA levels found in control untreated siNT-transfected cells and are the means ± SEM of at least three independent assays. *, $p < 0.05$.

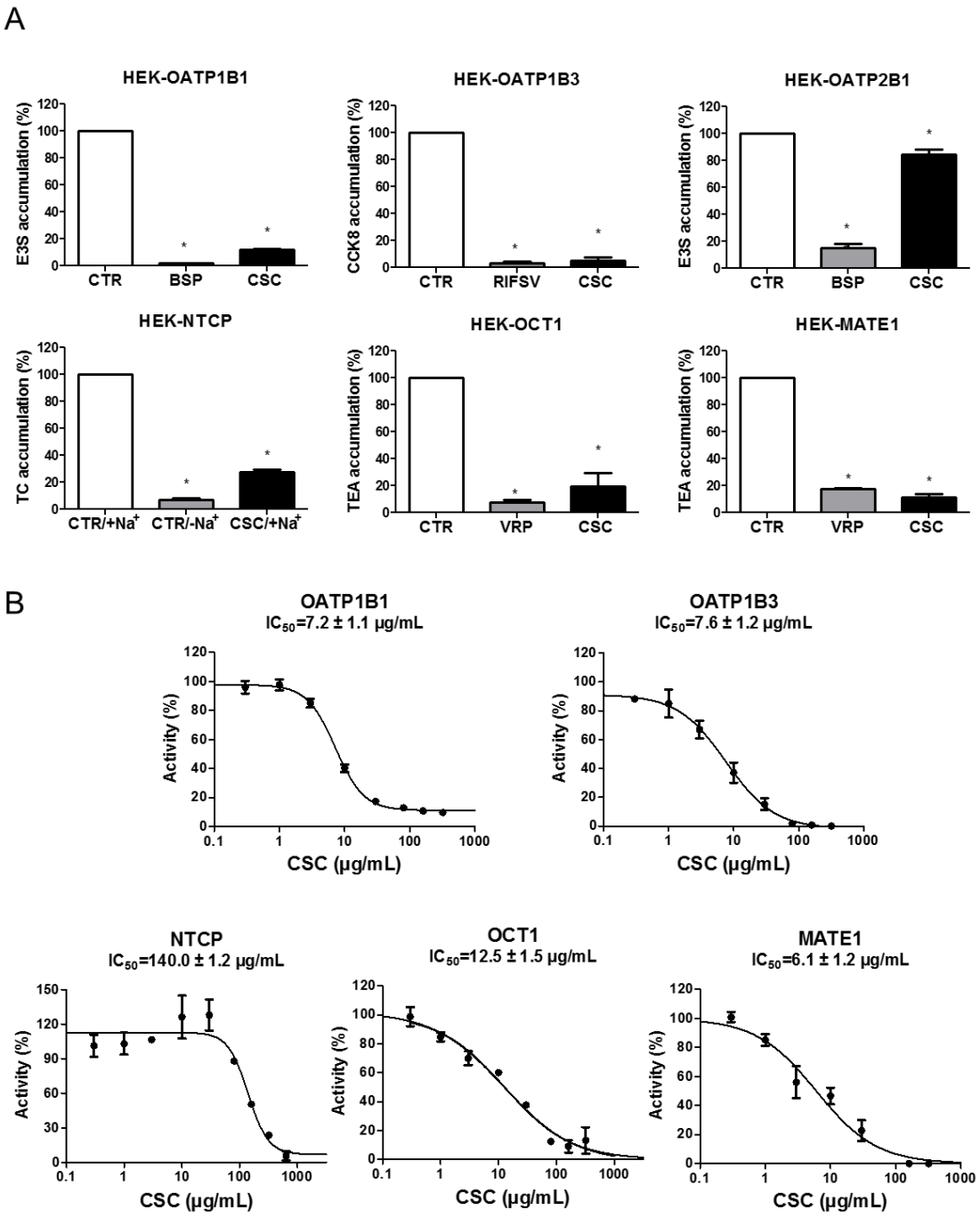

Figure 1

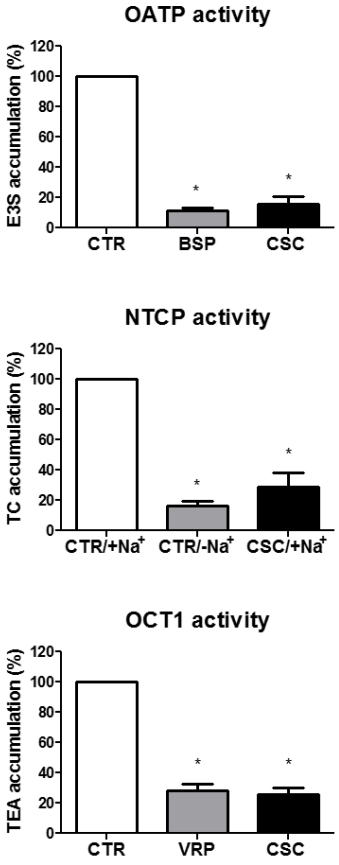

Figure 2

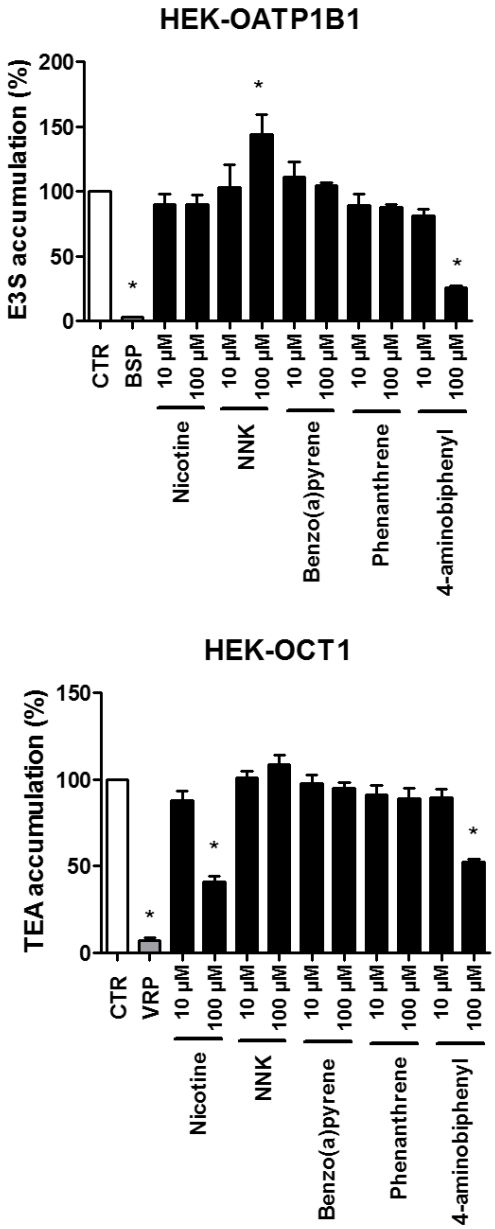

Figure 3

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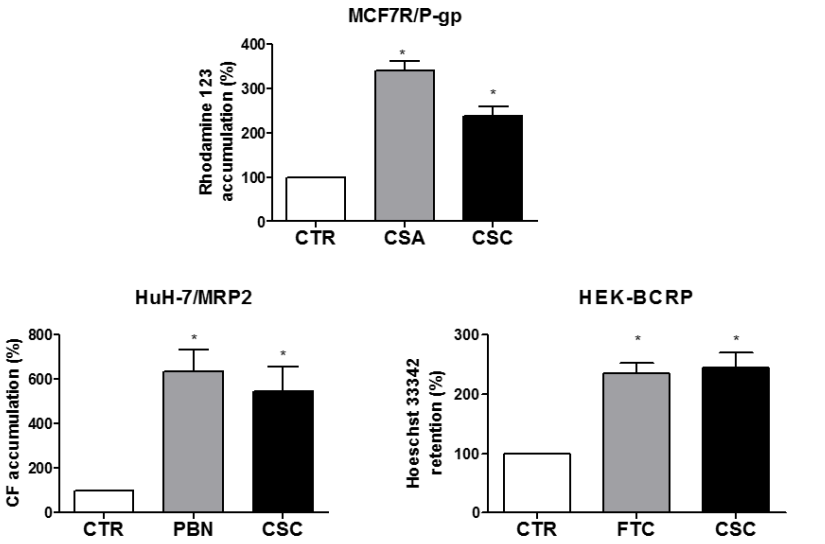

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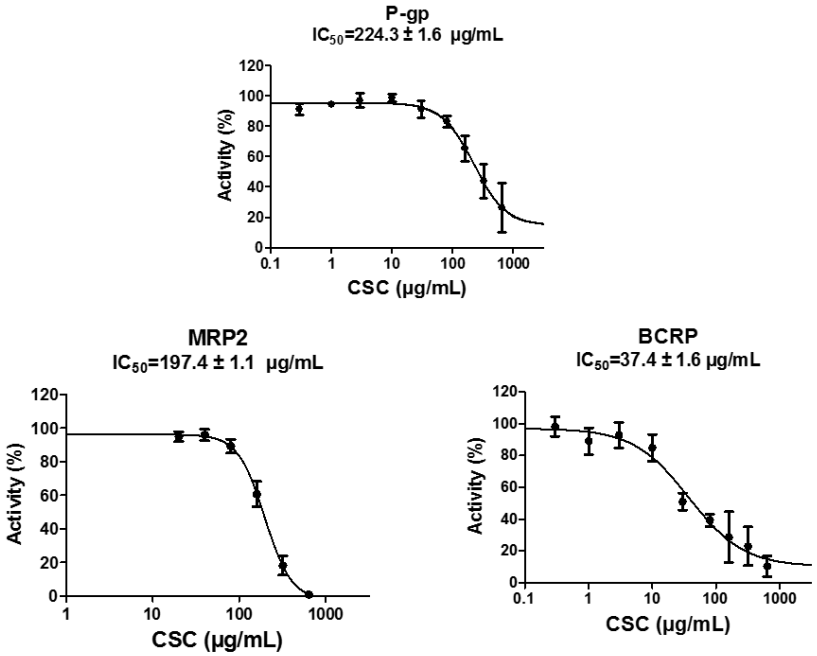

Figure 4

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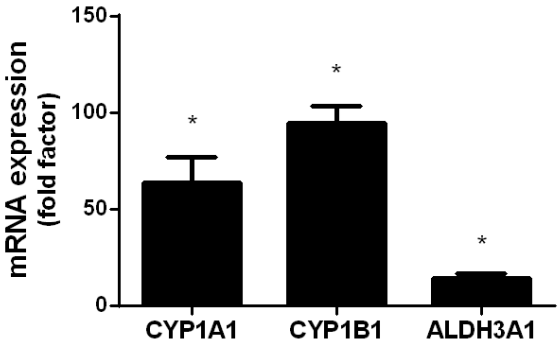

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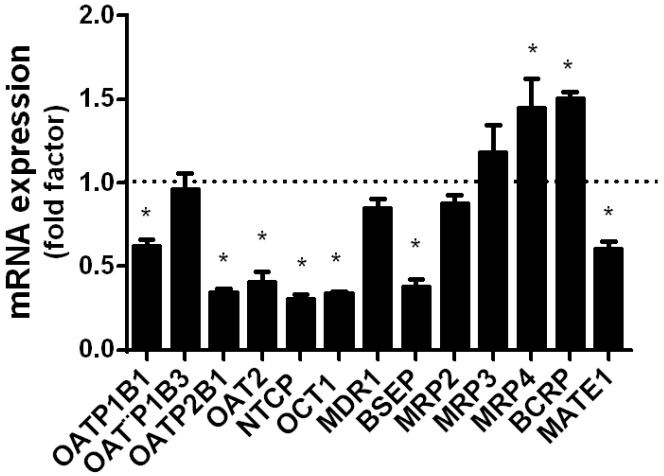

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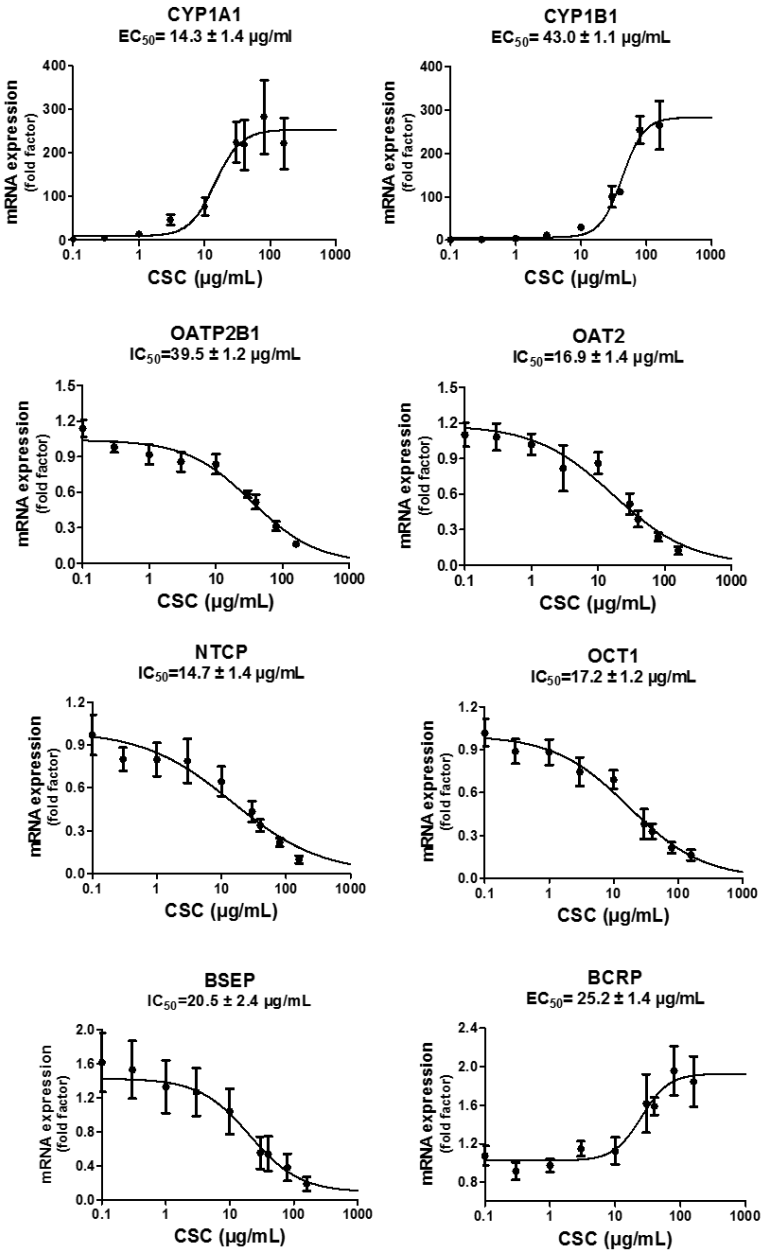

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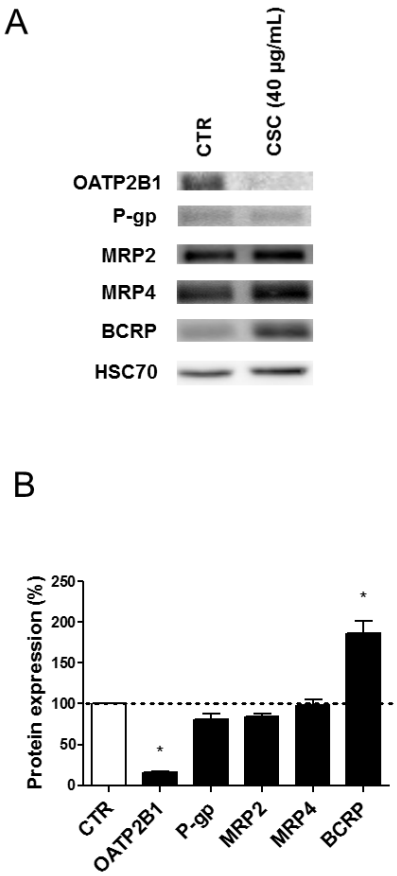

Figure 7

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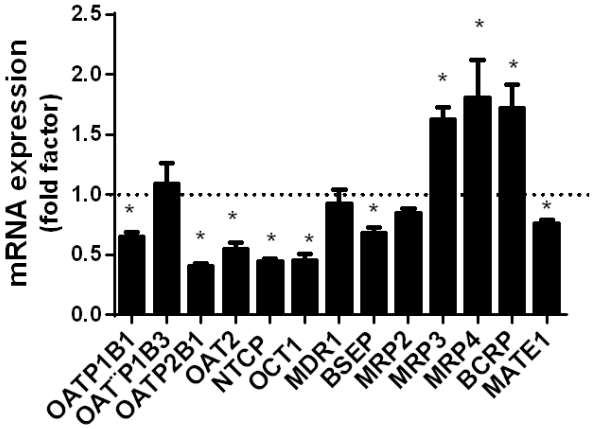

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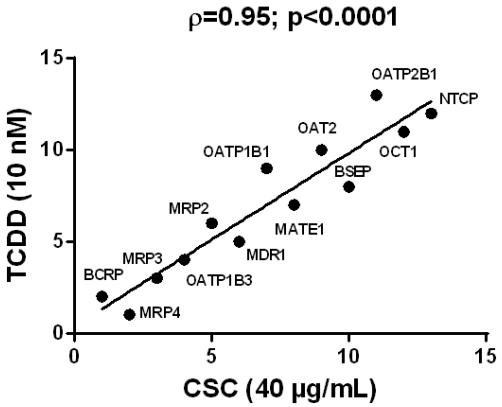

Figure 8

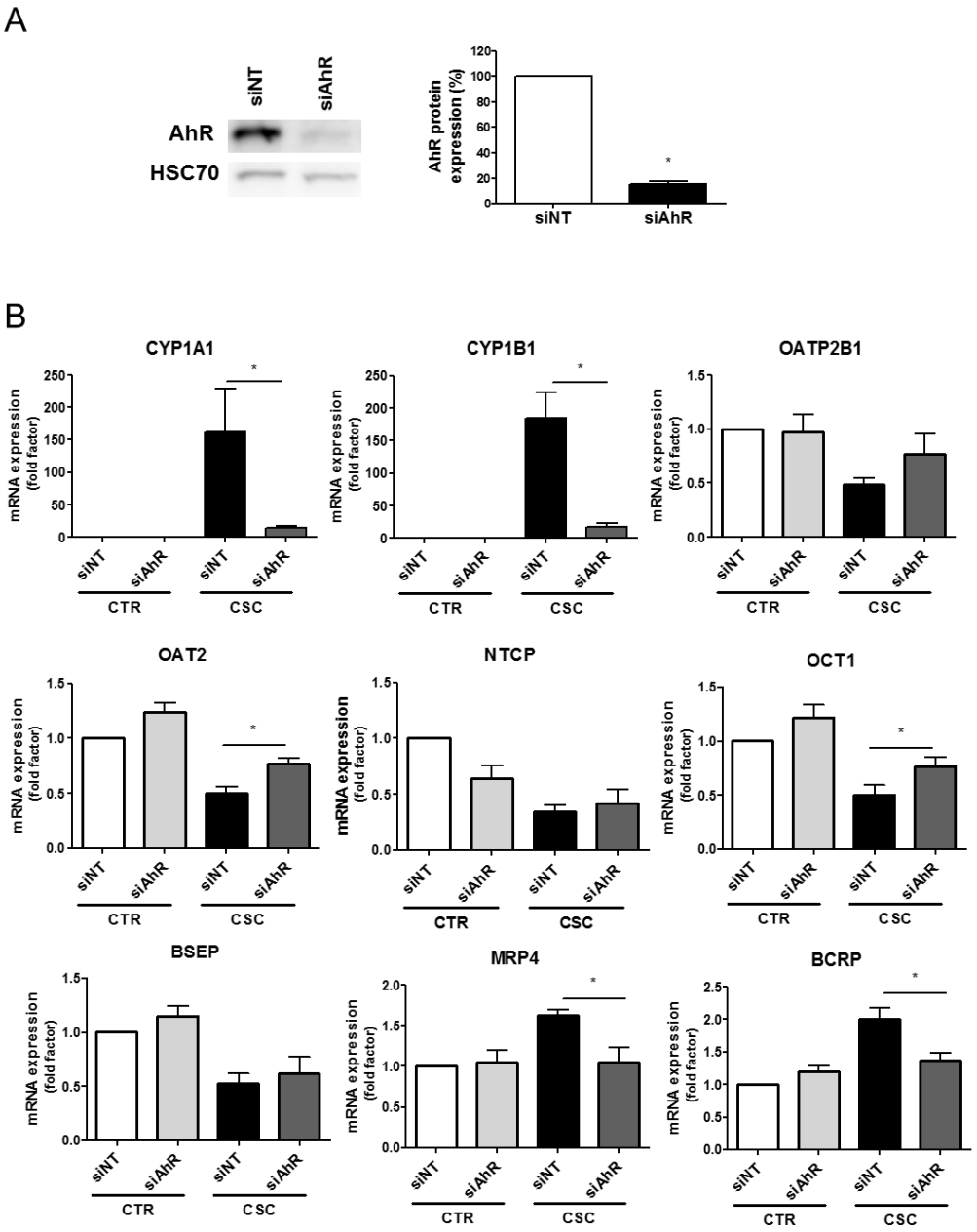

Figure 9

Table 1: Summary of CSC effects on hepatic drug transporter activity and expression

[a]strong inhibition: IC_{50} values in the 6-37 µg/mL range for CSC; moderate inhibition: IC_{50} values in the 140-224 µg/mL range for CSC; marginal inhibition: inhibition by less than 50% for 320 µg/mL CSC.
[b]ND: not determined.

Transporter	Activity[a]	Expression	
		mRNA	*Protein*
OATP1B1	Strong inhibition	Repression	ND[b]
OATP1B3	Strong inhibition	No change	ND
OATP2B1	Marginal inhibition	Repression	Repression
OAT2	ND	Repression	ND
NTCP	Moderate inhibition	Repression	ND
OCT1	Strong inhibition	Repression	ND
MATE1	Strong inhibition	Repression	ND
MDR1/P-gp	Moderate inhibition	No change	No change
BSEP	ND	Repression	ND
MRP2	Moderate inhibition	No change	No change
MRP3	ND	No change	ND
MRP4	ND	Induction	No change
BCRP	Strong inhibition	Induction	Induction